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Hofmann, Andreas ; Thiesler, Thore ; Gerrits, Bertran ; Behnke, Silvia ; Sobotzki, Nadine ; Omasits, Ulrich ; Bausch-Fluck, Damaris ; Bock, Thomas ; Aebersold, Ruedi ; Moch, Holger ; Tinguely, Marianne ; Wollscheid, Bernd

Abstract: PURPOSE: Classical Hodgkin lymphoma (cHL) is characterized by a low percentage of tumor cells in a background of diverse, reactive immune cells. cHL cells commonly derive from preapoptotic germinal-center B cells and are characterized by the loss of B cell markers and the varying expression of other hematopoietic lineage markers. This phenotypic variability and the scarcity of currently available cHL-specific cell surface markers can prevent clear distinction of cHL from related lymphomas. EXPERIMENTAL DESIGN: We applied the Cell Surface Capture (CSC) technology to directly measure the pool of cell surface exposed proteins in four cHL and four non-Hodgkin lymphoma (NHL) cell lines. RESULTS: More than 1 000 membrane proteins, including 178 CD annotated proteins, were identified and allowed the generation of lymphoma surfaceome maps. The functional properties of identified cell surface proteins enable, but also limit the information exchange of lymphoma cells with their microenvironment. CONCLUSIONS AND CLINICAL RELEVANCE: Selected candidate proteins with potential diagnostic value were evaluated on a tissue microarray (TMA). Primary lymphoma tissue of 126 different B cell derived lymphoma cases were included in the TMA analysis. The TMA analysis indicated gamma-glutamyltranspeptidase 1 as a potential additional marker that can be included in a panel of markers for differential diagnosis of cHL versus NHL.

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Surfaceome of Classical Hodgkin and Non-Hodgkin Lymphoma

Andreas Hofmann,[1,2] Thore Thiesler,[3] Bertran Gerrits,[4] Silvia Behnke,[3] Nadine Sobotzki,[1,5] Ulrich Omasits,[1] Damaris Bausch-Fluck,[1,5] Thomas Bock,[1] Ruedi Aebersold,[1,6] Holger Moch,[3] Marianne Tinguely,[3*] and Bernd Wollscheid[1,5*]

[1]Department of Biology, Institute of Molecular Systems Biology, Swiss Federal Institute of Technology (ETH) Zurich, Auguste-Piccard-Hof 1, 8093 Zurich, Switzerland,
[2]Ph.D. Program in Molecular Life Sciences, University of Zurich (UZH)/ ETH Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland,
[3]Institute of Surgical Pathology, University Hospital Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland,
[4]Functional Genomics Center Zurich, UZH/ ETH Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland,
[5]Department of Health Sciences and Technology, ETH Zurich, Auguste-Piccard-Hof 1, 8093 Zurich, Switzerland,
[6]Faculty of Science, UZH, Winterthurerstrasse 190, 8057 Zurich, Switzerland

[*]Corresponding authors:
Bernd Wollscheid, ETH Zurich, IMSB HPT-D77, Auguste-Piccard-Hof 1, 8093 Zurich, Switzerland, wbernd@ethz.ch
and
Marianne Tinguely, Institute of Surgical Pathology, University Hospital Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland, marianne.tinguelykovarik@uzh.ch

Running title:
MS-BASED PHENOTYPING

Abbreviations:
The abbreviations used are: cHL, classical Hodgkin lymphoma; CD, cluster of differentiation; CSC technology, Cell Surface Capture technology; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; HL, Hodgkin lymphoma; HRS, Hodgkin-Reed-Sternberg; MALTL, mucosa-associated lymphoid tissue lymphoma; NHL, non-Hodgkin lymphoma; NLPHL, nodular lymphocyte-predominant Hodgkin lymphoma; TMA, tissue microarray

Keywords:
Classical Hodgkin lymphoma, Cell Surface Capture (CSC) technology, surfaceome, mass spectrometry-based phenotyping, cell surface proteins

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Statement of clinical relevance

Classical Hodgkin lymphoma (cHL) commonly derives from preapoptotic germinal-center B cells. cHL cells are characterized by the loss of B cell markers and the varying expression of other hematopoietic lineage markers. This phenotypic variability and the lack of distinctive markers for cHL can prevent clear differentiation from related lymphomas.

In this study, we combined complementary Cys-Glyco-CSC, Glyco-CSC and Lys-CSC technology variants for the in depth identification of cell surface proteins in cHL and non-Hodgkin lymphoma (NHL) cell lines seeking for distinctive patterns in cHL. Although, cHL and NHL cell lines have a similar overall composition of functional membrane protein groups, hierarchical clustering of the generated cell surfaceome maps separated cHL from NHL. Furthermore, B cell derived cHL cell lines were separated from T cell derived cHL cell lines in the hierarchical cluster analysis. To evaluate the potential of cell surface proteins for differentiation of cHL and NHL in a routine diagnostic setting, selected candidate markers were tested on tissue microarrays (TMAs). The TMA analysis indicated gamma-glutamyltranspeptidase 1 as a distinctive marker for cHL, which might be useful in an extended marker panel for the differential diagnosis of cHL.

Abstract

Purpose

Classical Hodgkin lymphoma (cHL) is characterized by a low percentage of tumor cells in a background of diverse, reactive immune cells. cHL cells commonly derive from preapoptotic germinal-center B cells and are characterized by the loss of B cell markers and the varying expression of other hematopoietic lineage markers. This phenotypic variability and the scarcity of currently available cHL-specific cell surface markers can prevent clear distinction of cHL from related lymphomas.

Experimental design

We applied the Cell Surface Capture (CSC) technology to directly measure the pool of cell surface exposed proteins in four cHL and four non-Hodgkin lymphoma (NHL) cell lines.

Results

More than 1 000 membrane proteins, including 178 CD annotated proteins, were identified and allowed the generation of lymphoma surfaceome maps. The functional properties of identified cell surface proteins enable, but also limit the information exchange of lymphoma cells with their microenvironment.

Conclusions and clinical relevance

Selected candidate proteins with potential diagnostic value were evaluated on a tissue microarray (TMA). Primary lymphoma tissue of 126 different B cell derived lymphoma cases were included in the TMA analysis. The TMA analysis indicated gamma-glutamyltranspeptidase 1 as a potential additional marker that can be included in a panel of markers for differential diagnosis of cHL versus NHL.

1 Introduction

Hodgkin lymphoma (HL) is one of the most frequent lymphomas in the Western world and it can be divided into classical HL and nodular lymphocyte-predominant HL (NLPHL). cHL accounts for 95% of the HL cases and it is characterized by a minority of neoplastic Hodgkin-Reed-Sternberg (HRS) cells and an extensive inflammatory cellular infiltrate [1, 2]. HRS cells usually derive from preapoptotic germinal-center B cells, and a small proportion of approximately 1-2% of cHL possibly originates from T cells [3, 4]. The differential diagnosis of cHL relies on a combination of morphology, immunophenotype, and genetic alterations according to the WHO classification [5]. The immunophenotype of HRS cells is characterized by the global loss of B cell markers and the aberrant expression of markers of other lineages. The limited number of known consistently expressed markers in cHL limits the number of markers included in panels for differential diagnosis. The limited immunophenotype can hamper distinction between cHL and related diseases, such as NLPHL, diffuse large B cell lymphomas (DLBCL) and their variant T cell rich large B cell lymphoma, and anaplastic large cell lymphoma. The overlapping phenotypes of these related diseases lead to so-called “grey zone” lymphomas, which are difficult to unambiguously classify [6]. Thus, extended panels with more markers are required for the accurate classification and sub-classification of lymphomas. Large scale investigation of surface markers in primary cHL is hampered by the scarcity of tumor cells. Therefore, better knowledge of the phenotype of cHL cells, obtained by the analysis of the surfaceome, may improve the understanding of cHL pathobiology and aims at facilitating the unambiguous classification as a base for a disease specific therapy.

Mass spectrometry (MS) based proteomics enables the multiplexed identification and quantification of peptides in a discovery-driven analysis of the proteome [7]. Proteomic technologies are expected to revolutionize clinical practice but are not yet routinely applied for hematological malignancies [8]. Therefore, the application of proteomic technologies to cHL is still limited. Previous studies were either focused on the secretome of HL cells [9] or carried out using 2-dimensional gel electrophoresis that commonly leads to an underrepresentation of hydrophobic cell surface proteins [10-12]. Furthermore, Wallentine et al. extensively analyzed by LC-MS three different subcellular fractions of

two cHL cell lines and identified several hundred proteins per fraction [13]. However, membrane proteins were also in this study the smallest group of identified proteins. A recent, extensive shotgun proteomics study analyzed in-depth DLBCL cell lines, but included two cHL cell lines only as a reference [14].

The analysis of the cell surface proteome by MS is hampered by factors, such as the hydrophobicity of transmembrane proteins and the low relative abundance of cell surface proteins compared to intracellular proteins. We circumvented to a large extent these issues by developing the MS-based CSC technology [15], which was extended with complementary CSC technology variants [16]. Here, we applied the Cys-Glyco-CSC, Glyco-CSC and Lys-CSC technology variants for the multiplexed identification of cell surface proteins and the generation of surfaceome maps of cHL and NHL cells. By analyzing four cHL cell lines and four B cell derived NHL cell lines, we identified overall more than 1 000 membrane proteins, including 178 CD annotated proteins. Detailed surfaceome map analysis revealed surfaceome plasticity in-between the analyzed cell types, indicating potential diagnostic value. To test differentially expressed proteins between cHL and NHL with antibody-based probes, we created a TMA that contained 126 different patient lymphoma cases. The primary tissue spots on the TMA were derived from four different lymphoma subtypes, which allowed to readily assess the suitability of new cell surface markers to be included in an extended panel of markers for a better differential diagnosis of cHL versus NHL.

2 Materials and methods

Cell culture

The cHL cell lines HDLM2, KMH2, L428, and L540 were kindly provided by Prof. Dr. Bernhard Odermatt (Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland) and the NHL cell lines HBL1, SUDHL4, SUDHL6, and U2932 were kindly provided by Dr. Liza Ho (Institute of Pathology, University of Geneva, Geneva, Switzerland). All cell lines were cultured in RPMI1640 (5% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin; Sigma-Aldrich, Buchs, Switzerland) at 37 °C and 5% CO_2.

CSC technology and LC-MS analysis

Peptides of cell surface proteins were isolated with the Cys-Glyco-CSC/ Glyco-CSC and Lys-CSC technology and analyzed by LC-MS as described previously [16] and in the supplemental materials and methods.

MS data analysis

Raw data files from the MS instrument were converted with ReAdW into mzXML files [17] and mzXML files were searched with Sorcerer™-SEQUEST® [18] (version 3.5) against a concatenated protein database combining the human protein database of the UniProtKB/Swiss-Prot Protein Knowledgebase (version 56.9), the reversed sequences of all proteins, and common contaminants (41,104 protein entries). The database search criteria included: 40 ppm mass tolerance for the precursor ion, 0.5 Da mass tolerance for fragment ions, variable modifications of 0.984020 Da for asparagines (representing formerly N-glycosylated asparagines after deamidation through the PNGase F treatment), 145.019745 Da for lysines (representing 3-(carbamidomethylthio)propanoyl-tagged lysines), and 15.994920 Da for methionines (representing oxidized methionines), carbamidomethylation as static modification for cysteines, at least one tryptic terminus (K, R), and up to two missed cleavage sites. Statistical analysis for all LC-MS analyses of each cell line was performed using the Trans-Proteomic Pipeline TPP [19] v4.0 JETSTREAM rev 2 including PeptideProphet [20] and ProteinProphet [21]. Peptides with a PeptideProphet probability score of $p \geq 0.05$ were included in the subsequent ProteinProphet analysis. The ProteinProphet probability score was set to ≥ 0.9, which resulted in an average protein false discovery rate of less than 1% for all LC-MS analyses for each cell line estimated by ProteinProphet. Proteins, which were only identified with single peptide identifications in the whole data set, were excluded from subsequent analyses. If peptide identifications led to an indistinguishable group of proteins, the first protein identifier was used for subsequent analyses and the remaining protein identifiers were listed in the supplemental tables. For the final complete data set with all cell lines, a protein false discovery rate of less than 2% and a peptide false discovery rate of less than 1% were estimated by the MAYU software [22]. The Skyline software [23] was used to create from the pepXML and mzXML files a spectral library containing MS/MS spectra with a cut-off score of 0.05. The sequence coverage of identified proteins

was determined with the Protein Coverage Summarizer (version 1.2.3365), which was downloaded from the website of the Biological MS Data and Software Distribution Center of the Pacific Northwest National Laboratory (Richland, WA, USA). CD numbers were mapped to UniProtKB/Swiss-Prot IDs according to the UniProtKB/Swiss-Prot Protein Knowledgebase cross-reference table (version 57.0). Transmembrane helices of proteins were predicted with two algorithms, the TMHMM algorithm [24] (version 2.0) and the Phobius algorithm [25]. Protein identifications were denoted as membrane proteins, if at least one transmembrane helix algorithm predicted one or more transmembrane helices for the protein sequences. Furthermore, CD annotated proteins and proteins, which contained a lipid moiety-binding region according to the UniProtKB/Swiss-Prot Protein Knowledgebase, were also denoted as membrane proteins. Molecular functions, biological processes and pathways of identified proteins were assigned by the PANTHER (Protein ANalysis THrough Evolutionary Relationships) database [26] (version 6.1.1) based on the alphabetically first UniProtKB/Swiss-Prot AC number. Hierarchical cluster analysis was carried out with the Spotfire DecisionSite 9.1.1 software (TIBCO Software Inc., Palo Alto, CA, USA) based on protein identifications in the different cell lines. Unweighted pair-group method with arithmetic mean was chosen for clustering method and Euclidean distance for similarity measure. Proteins were excluded from the cluster analysis, if the total sum of assigned spectra over all cell lines was less than 10 spectra.

Patients

Formalin fixed, paraffin embedded tissue biopsies of 144 patients with cHL (34 patients), FL (follicular lymphoma, 36 patients), DLBCL (36 patients), and MALTL (mucosa-associated lymphoid tissue lymphoma, 38 patients), which were diagnosed between 1998 and 2006, were retrieved from the files of the Institute of Surgical Pathology, University Hospital Zurich, Switzerland. Tissue cores of 18 patients were excluded (4 patients with cHL, 5 patients with FL, 3 patients with DLBCL, and 6 patients with MALTL) due to loss of tumor tissue caused by the different cylinder lengths of the donor cores. All tissue cores on the TMA were typical representatives of the respective lymphoma subtypes. All cHL expressed CD30 and Pax5 indicating a B cell origin. There were no “grey zone” lymphomas included and all DLBCL were negative for CD30. The baseline characteristics of all

investigated subjects are shown in (supplemental Table S1). The study was approved by the Ethical Committee of the Canton of Zurich (StV 2-2007).

Immunohistochemistry

TMAs were constructed with 0.6 mm diameter cores of patient tissue as previously described [27]. TMAs were dewaxed in xylene, rehydrated through a graded ethanol series and washed in phosphate buffered saline. Antigen retrieval was achieved by heat treatment in a pressure cooker at 110 °C in citrate buffer (pH 6). Endogenous peroxidase activity was blocked by incubation in 0.6% H_2O_2 in 1:1 (v/v) methanol-water for 10 minutes at room temperature. Subsequently, TMAs were incubated with protein blocking solution (Dako, Glostrup, Denmark) for 30 minutes at room temperature. Thereafter, slides were incubated with the appropriate dilution of primary antibody (supplemental Table S2). Immunodetection was performed with EnVision™+ Dual Link System-HRP (Dako) employing 3,3'-diaminobenzidine/ H_2O_2 as chromogen. Sections were counterstained with hematoxylin. All biopsies were reviewed by two pathologists (M.T. and T.T.). The staining intensity for all proteins investigated was assessed in a four tiered system and graded as negative (-), weak (1+), moderate (2+), and strong (3+) [28]. Biopsies with weak, moderate or strong staining were stated as positive and biopsies with no staining were stated as negative. TMAs were scanned in 40x mode on a NanoZoomer (Hamamatsu Photonics, Shizuoka, Japan) and images were acquired using the web-based Distiller software (SlidePath, Dublin, Ireland). High magnification images were acquired on a Leica DM IRBE microscope (63x/0.7, Leica objective lenses) by a QImaging Retiga 1300 camera using QCapture v2.68.6 software (QImaging, Surrey, Canada). Contrast of images was adjusted with Adobe Photoshop CS4.

3 Results

Generation of surfaceome maps of cHL and NHL

Here, the surfaceome of four different cHL cell lines was analyzed with complementary CSC technology variants. Two of the cHL cell lines represent B cell derived cHL cell lines, KMH2 and L428, respectively [29, 30]. The other two cHL cell lines represent T cell derived cHL cell lines, HDLM2 and L540, respectively [31, 32]. The four cHL cell lines were compared with four B cell derived NHL cell lines. Two of the NHL cell lines represent activated B-like DLBCL cell lines, HBL1 and U2932, respectively [33, 34]. The other two NHL cell lines represent germinal center B-like DLBCL cell lines, SUDHL4 and SUDHL6, respectively [35]. By applying the CSC technology, peptides from extracellular domains of cell surface proteins were isolated and identified by MS (Fig. 1). Several hundred membrane proteins were identified with the CSC technology in each lymphoma cell line (Table I, supplemental Table S3). Overall, more than 1 000 membrane proteins, including 178 bona fide cell surface exposed CD proteins, were identified in the eight lymphoma cell lines (Fig. 2A; supplemental Table S4-6). cHL and NHL cells shared the majority of identified membrane proteins (57.7%) and CD proteins (54.5%). However, 459 membrane proteins were only identified in one lymphoma group, suggesting clear differences between cHL and NHL cells based on the CSC analyses.

Functional protein classes in cHL and NHL

Different classes of cell surface proteins are required for a cell to sense its microenvironment and to respond to external stimuli. Hence, proteins present at the cell surface reflect functional capacities of the cell. Categorization into molecular functions of all membrane proteins identified in cHL and NHL cell lines, respectively, showed a similar distribution of functional protein classes between the two lymphoma groups (Fig. 2B; supplemental Table S7). The largest functional protein groups included receptor proteins, defense/immunity related proteins, transporters, and signaling molecules. The number of spectra assigned to an identified protein can be used for the estimation of protein abundance [36]. The top three proteins identified with the highest number of spectra in all eight lymphoma cell lines were CD45, CD205, and CD54 for receptors, HLA class I, HLA class II, and

CD46 for defense/immunity related proteins, CD102, CD59, and CD37 for signaling molecules, and CD298, cholin transporter-like protein (CTL2), and neutral amino acid transporter A (SATT) for transporters. The overlapping proteins found in cHL and in NHL cell lines showed a strong enrichment of defense/immunity related proteins, which are depleted in the group of non-overlapping proteins between cHL and NHL (supplemental Fig. S1). In summary, the CSC analyses suggest that cHL and NHL cells have a similar overall composition of functional membrane protein groups and have high abundant proteins in common.

Comparative surfaceome map analysis of cHL and NHL

Although the distributions of functional protein groups were very similar for cHL and NHL, the comparison of identified proteins showed that the composition of each functional protein group differed between cHL and NHL. For example, peptides of the receptors CD30, IL18R, and CRLF2, respectively, were only identified in cHL and not in NHL cell lines. CD30 was identified in each cHL cell line with more than 100 assigned spectra with the CSC technology (supplemental Table S4). In combination with other receptors, CD30 is important for the activation of the NF-κB, PI3K/Akt, and MAPK/Erk signaling cascade [1, 2]. The CSC technology readily identified the CD30 protein, which is a hallmark of HRS cells and it is commonly used as a marker for the differential diagnosis of cHL. Cytokine receptors in HRS cells activate the JAK/STAT signaling pathway and signaling of IL13R and IL21R are known to be important for proliferation of HRS cells [37, 38]. Interestingly, in addition to IL13R and IL21R, several other cytokine receptors including IL18R and CRLF2 were identified in cHL cells with the CSC technology.

The surfaceome analyses with the CSC technology showed differences in protein identifications not only between cHL and NHL cell lines but also within the group of cHL cell lines. Several receptors and receptor ligands that are crucial components in different signal transduction pathways were identified only in the two B cell derived cHL cell lines, KMH2 and L428. For example, two receptor tyrosine kinases, EphB2 (EPHB2) and the previously described CD136 (RON) [39], were identified only in B cell derived cHL cell lines. Interestingly, Notch1 (NOTC1) and Notch2 (NOTC2) were identified in all eight lymphoma cell lines, but the two transmembrane and GPI-anchored, respectively, Notch ligands, Jagged1 (JAG1) and Contactin1 (CNTN1), were identified only in the B cell derived

cHL cell lines. In the T cell derived cHL cell lines, HDLM2 and L540, several T cell antigens were identified including CD2, CD4, and CD90. However, these T cell antigens were not consistently and not exclusively identified in the T cell derived cHL cell lines. These results show the highly variable phenotype of cHL. Nonetheless, hierarchical cluster analysis of identified membrane proteins with more than 10 assigned spectra led to a separation of B cell derived from T cell derived cHL cell lines and also to a separation of cHL from NHL cell lines (Fig. 2C; supplemental Table S8-10), which suggests that the membrane protein expression profile is characteristic for lymphoma subgroups.

In addition to proteins exclusively identified in only one lymphoma group or lymphoma subgroup, many proteins were identified with different numbers of assigned spectra between the two lymphoma groups indicating a difference in protein abundances. An interesting example is the gamma-glutamyltranspeptidase 1 (GGT1, CD224), which is widely used as a diagnostic marker for liver disease. GGT1 was identified overall with 142 spectra in the four cHL cell lines but with less than 10 spectra in only one NHL cell line (supplemental Fig. S2, supplemental Table S4). Already in the 1980s, it was shown cytochemically that HRS cells exhibit a very strong GGT1 activity [40, 41], but ever since GGT1 expression in HRS cells did not attract a lot of attention in HL research. The hepatocyte growth factor receptor (MET) that is encoded by the proto-oncogene c-met was also identified with a large quantitative difference of assigned spectra between cHL and NHL cells. MET was previously described to be expressed in HRS cells [42] and the CSC technology identified MET predominantly in cHL cell lines with a difference of 288 assigned spectra compared with 12 assigned spectra for the protein identification in cHL and NHL cells, respectively (supplemental Fig. S2, supplemental Table S4). Besides the aberrant expression of cell surface proteins, B cell derived HRS cells are characterized by the loss of B cell markers [1, 2]. Consistent with this expectation, the B cell markers, CD19, CD20, CD22, CD79a, and CD79b, were not identified with the CSC technology in the cHL cell lines but were identified in all NHL cell lines (Fig. 3).

In summary, these examples of identified proteins show that the CSC-based surfaceome mapping enabled an extensive phenotyping of cHL and NHL cell lines far beyond what is usually achieved with antibody-based technologies. Furthermore, the comparative analysis reliably recapitulated known

phenotypic characteristics of cHL cells and also identified cell surface proteins with little prior information about their functions in cHL.

Tissue microarray analysis

Additional cHL markers that extend the current marker panel may significantly contribute to facilitate and bolster the reliable diagnosis of cHL. In order to investigate if identified proteins by LC-MS are useful for the differential diagnosis of cHL versus other B cell derived hematological malignancies, in-house developed TMAs were immunohistochemically analyzed for the expression of candidate classification markers. The TMAs contained 126 different lymphoma cases including 30 cHL, 33 DLBCL, 31 FL, and 32 MALTL cases with different patient characteristics (supplemental Table S1). From numerous tested antibodies, only two paraffin-reactive antibodies were identified that showed reliable staining properties. The candidate classification markers, GGT1 and MET, were identified by LC-MS predominantly in cHL cell lines (supplemental Fig. S2). Dilutions of antibodies against the two candidate classification markers were optimized on a cell line microarray containing all eight lymphoma cell lines that had been analyzed with the CSC technology (supplemental Table S2). Then, TMAs were analyzed immunohistochemically for the expression of GGT1 and MET, respectively (Fig. 4). Non-informative arrayed tissue samples due to missing tissue or lack of tumor cells in the arrayed tissue cores were excluded. Expression of all two candidate classification markers was detected in HRS cells with a cytoplasmic and membranous staining (Fig. 4B-C). GGT1 was predominantly expressed in cHL with 100% positive cHL, 27% positive DLBCL, 3% positive FL, and 3% positive MALTL cases (Table II). The expression of MET was more variable with several positive cases in all four lymphoma groups. Thus, expression of both candidate classification markers derived from the proteomic comparison of cHL and NHL cell lines were detected in primary patient samples.

4 Discussion

Cell surface proteins are readily accessible and well suited as diagnostic and therapeutic targets. Currently, the panel of protein markers used for differential diagnosis of cHL is still limited. This lack of classification markers is partially due to difficulties to comprehensively compare neoplastic cells on the protein level. Here, we show that the CSC technology enabled the generation of surfaceome maps of cHL and NHL cell lines to a depth of more than 1 000 membrane proteins, including 178 CD proteins. Hierarchical cluster analysis of protein identifications led to a separation of cHL from NHL cell lines and to a separation of B cell derived from T cell derived cHL cell lines (Fig. 2C), which suggests that the membrane protein expression profile is characteristic for lymphoma subgroups. Furthermore, the differential surfaceome map analysis identified in cHL and NHL cell lines numerous receptors that represent crucial components of different signal transduction pathways, such as the NF-κB, PI3K/Akt, MAPK/Erk, JAK/STAT, and Wnt/beta catenin signaling pathway.

Comparative analysis of identified proteins in cHL and NHL cell lines reliably recapitulated known phenotypic characteristics of cHL. The loss of B cell markers and the expression of CD30, CD40, CD54, CD80, and CD274 [1, 2], to name several examples, were identified in the CSC-based surfaceome maps of cHL cells (Fig. 3). CD30 is commonly used as a marker for the differential diagnosis of cHL. More recently, CD30 is also used as a drug target by the antibody-drug conjugate brentuximab vedotin. Thus, the CSC technology enabled the reliable identification of the cHL marker CD30 that is exploited for diagnosis and targeted therapeutic intervention. The characteristic expression of CD15 in cHL, which is often used in marker panels for differential diagnosis of cHL, was not detected with our CSC-based protein identification approach, since CD15 represents the trisaccharide 3-fucosyl-N-acetyl-lactosamine, which is refractory to the applied peptide selection method.

Apart from proteins that are known to be expressed in cHL, numerous proteins with little prior information about their functional role in cHL were identified exclusively in cHL cell lines, such as CRLF2. CRLF2 is a type I cytokine receptor and it forms together with IL7R the heterodimeric receptor for thymic stromal lymphopoietin (TSLP) [43]. Interestingly, binding of TSLP to its receptor induces activation of STAT5 and STAT5 activity is known to induce in human tonsillar B cells

phenotypic changes similar to HRS cells [38, 43]. Thus, CRLF2 might play through STAT5 a role in Hodgkin lymphomagenesis [44]. It was also shown that CRLF2 overexpression is associated with poor outcome in B cell acute lymphoblastic leukemia [45].

Furthermore, proteins were identified with higher abundances based on spectral counting in cHL than in NHL cell lines, such as GGT1 and MET. GGT1 belongs to the family of gamma-glutamyl transferases (GGTs), which are post-translationally processed to form heavy and light chain containing single transmembrane spanning proteins. Drugs and carcinogens induce GGT expression in the liver and assays for GGT activity are widely used in the clinic to measure liver cell damage. GGTs are crucial for the metabolism of glutathione and the conversion of leukotriene C4 into leukotriene D4. Furthermore, there are indications that GGT also plays a crucial role in drug resistance of cancer cells [46]. The receptor tyrosine kinase MET is a disulfide-linked alpha/beta heterodimer and the beta-chain spans the plasma membrane. MET acts as a receptor for the hepatocyte growth factor, and it was speculated that ligand binding may lead to cytokine release in MET positive HRS cells [47]. MET is known to be important for the regulation of adhesion and migration of activated B cells and it is also expressed in germinal center centroblasts [48, 49]. In addition to the here in more detail discussed proteins, numerous other proteins were identified that represent interesting candidates to be tested for the differential diagnosis of cHL versus NHL, since they were identified either in only one lymphoma group or with a large quantitative difference between the lymphoma groups based on the number of assigned spectra for protein identifications.

Selected candidate classification markers, against which paraffin-reactive antibodies with reliable staining properties were available, were analyzed on TMAs containing 126 different lymphoma cases. The two candidate proteins for cHL, GGT1 and MET, were detected immunohistochemically in primary lymphoma tissue. GGT1 was detected in lymphoma cells of every single cHL patient but in contrast only in a minority of DLBCL patients, in a single FL patient and a single MALTL patient. This finding and the importance of GGT1 for the conversion of leukotriene C4 into leukotriene D4 might represent a very interesting link to the extensive inflammatory cellular infiltrate that is characteristic for cHL. Expression of MET was more heterogeneous and the limited number of patients on the TMA did not allow to deduce conclusive characteristics of MET positive patients.

In summary, we were able to in-depth analyze the cell surface proteome plasticity of cHL and NHL cell lines by applying the CSC technology. Our analysis identified new candidates that could be included in marker panels for the differential diagnosis of cHL versus NHL. A plethora of interesting protein identifications can be found in the data set, which could generate new hypotheses and represent the starting point for new studies to better understand the pathobiology of cHL and NHL. Prospective studies, including the analysis of a larger patient cohort with an extended marker panel that includes several of the candidate proteins, should contribute to more unambiguously differentiate cHL from NHL.

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Conflict of interest

The authors declare no competing financial interests.

Authorship

Contributions: A.H., R.A., H.M., M.T. and B.W. designed the research; A.H., T.T., B.G., S.B., N.S., D.B., T.B. and T.M. performed the research; A.H., U.O., T.T. and M.T. analyzed the data; A.H., T.T., M.T. and B.W. wrote the paper.

Correspondence: Bernd Wollscheid, Institute of Molecular Systems Biology, HPT D-77, ETH Zurich, Auguste-Piccard-Hof 1, 8093 Zurich, Switzerland; e-mail: wbernd@ethz.ch and

Marianne Tinguely, Institute of Surgical Pathology, University Hospital Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland; e-mail: marianne.tinguelykovarik@uzh.ch

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FIG. 1. **The CSC technology uses an affinity labeling strategy to enrich for peptides from the extracellular domains of cell surface proteins.** This enrichment strategy involves [1] complementary biotinylation of cell surface proteins with different reactive biotin derivatives, [2] cell homogenization and membrane isolation, [3] protein digestion, [4] streptavidin affinity enrichment of biotinylated peptides, [5] peptide release, [6] peptide analysis by LC-MS and peptide identification.

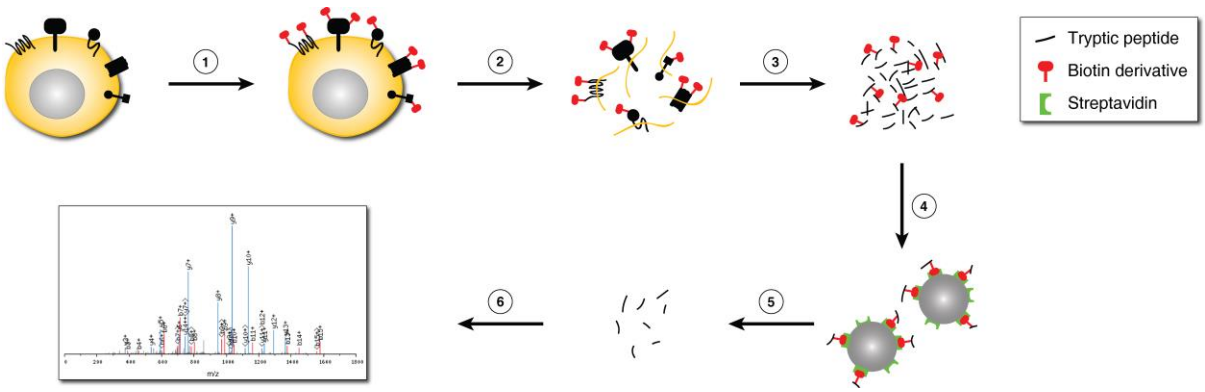


FIG. 2. **CSC analysis of cHL and NHL cell lines.** A) Venn diagrams showing overlapping membrane and CD protein identifications in cHL and NHL cells. B) Distribution of PANTHER molecular functions of identified membrane proteins in cHL and NHL cells. C) Hierarchical cluster analysis of identified membrane proteins in cHL and NHL cells. The hierarchical cluster analysis was restricted to 709 membrane proteins that were identified with at least 10 assigned spectra in the whole data set. Each row represents one protein and each column represents one cell line. Protein identifications or missing protein identifications are depicted in black and white, respectively. The distribution of CD annotated proteins is shown in red.

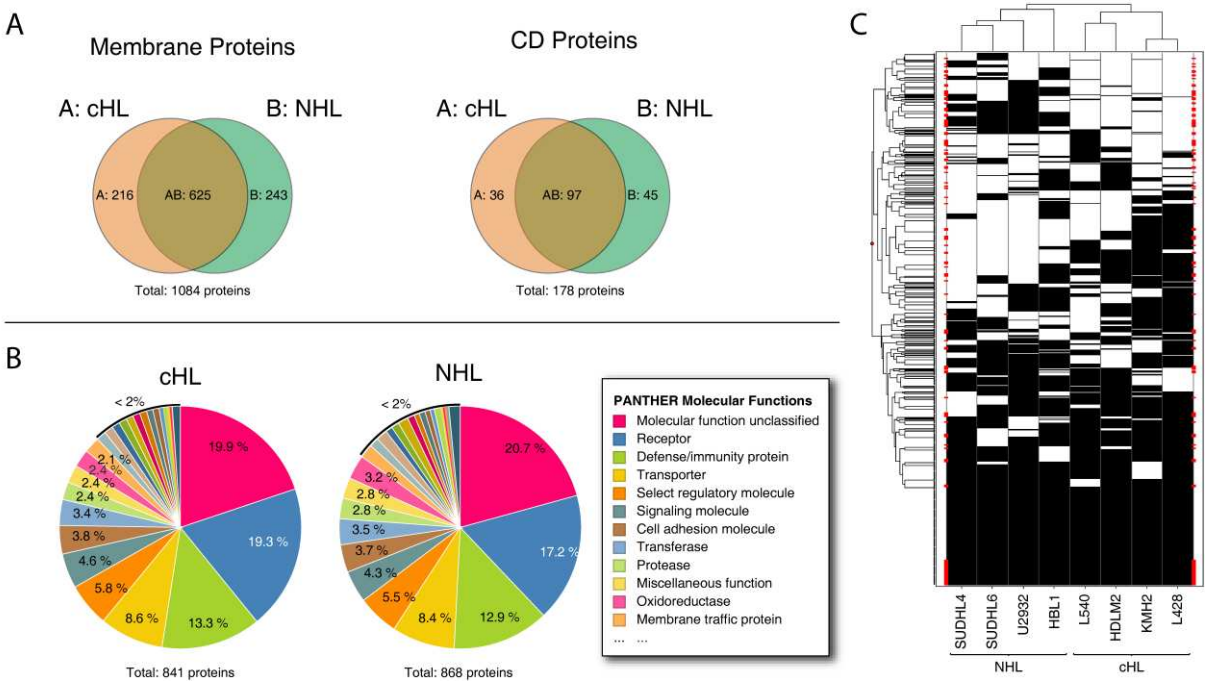
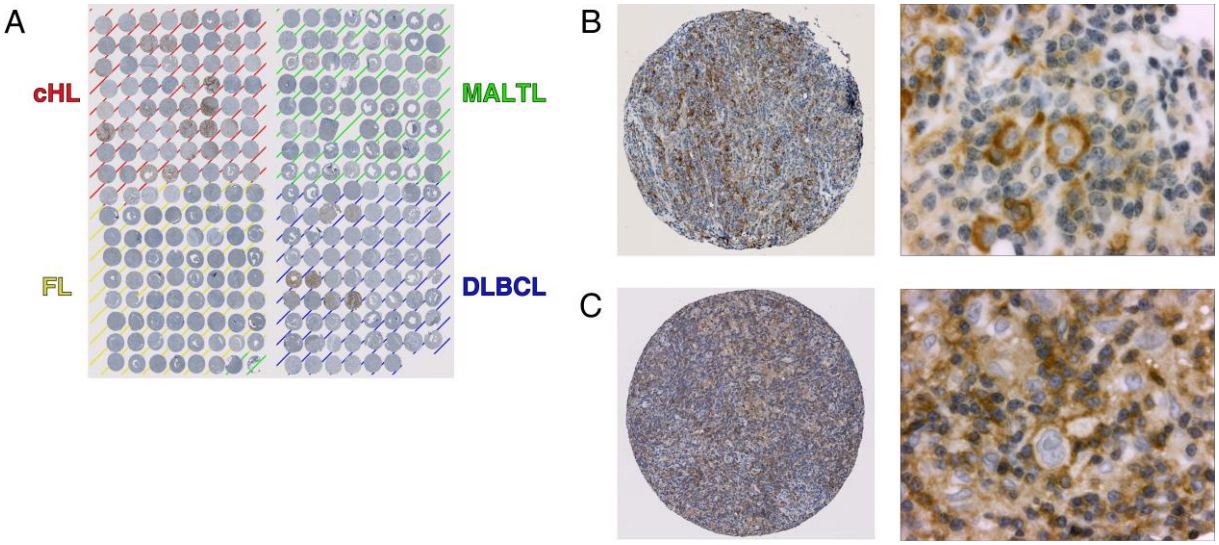


FIG. 3. **Extensive CD phenotyping of cHL and NHL cells.** Overview of 146 selected CD proteins, which were identified with a sum of at least 10 assigned spectra in the four cHL cell lines or in the four NHL cell lines. The inner color code of the squares visualizes the number of assigned spectra per CD protein for cHL cells (upper squares) and NHL cells (lower squares).

FIG. 4. **Immunohistochemical detection of GGT1 and MET in cHL.** (A) Schematic overview of the TMA containing tissue samples of 126 different lymphoma cases. (B) Immunostainings for GGT1 and (C) MET with hematoxylin counterstainings in HRS cells in low magnification (image on the left) and high magnification (image on the right).



TABLES

TABLE I

Number of identified membrane proteins and CD proteins in cHL and NHL cell lines

Cell line	Lymphoma	Membrane proteins	CD proteins
KMH2	cHL (B cell derived, MC)	536	94
L428	cHL (B cell derived, NS)	588	92
HDLM2	cHL (T cell derived, NS)	512	94
L540	cHL (T cell derived, NS)	459	94
HBL1	DLBCL (ABC)	586	97
U2932	DLBCL (ABC)	510	107
SUDHL4	DLBCL (GCBC)	464	83
SUDHL6	DLBCL (GCBC)	457	95

MC, mixed cellularity; NS, nodular sclerosis; ABC, activated B-like; GCBC, germinal center B-like

TABLE II
TMA analysis of GGT1 expression and MET expression in lymphoma patients

Lymphoma	GGT1			MET		
	n	N (%)	P (%)	n	N (%)	P (%)
DLBCL	33	24 (73)	9 (27)	33	23 (70)	10 (30)
FL	30	29 (97)	1 (3)	30	9 (30)	21 (70)
HL	28	0 (0)	28 (100)	25	8 (32)	17 (68)
MALTL	32	31 (97)	1 (3)	31	24 (77)	7 (23)

n, number of patients; N, negative; P, positive